Counteracting Inhibition of Apoptosis by c-Myc, Bcl-2, and c-FLIP:

A Multifaceted Approach to Inducing Cell Death

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ABSTRACT

Apoptosis is essential to normal growth and development. Discrepancies in the timing, location, and progression of apoptosis produce a number of conditions. More specifically, abnormalities in the expression of c-Myc, a transcription factor that regulates cell growth and development, have been correlated with cell proliferation. Expression of Bcl-2, a mitochondrial protein, antagonizes the apoptotic function of c-Myc. A multifaceted approach is introduced that seeks to inhibit c-Myc, Bcl-2, and c-FLIP. Synthetic nucleic acids, called aptamers, have been selected against a number of pathogenic proteins and are a promising means of inhibiting these protein targets. Aptamers bind with high specificity and high affinity to their protein targets and are powerful tools in diagnostics and therapeutics. Aptamers against c-Myc, Bcl-2, and c-FLIP are expected to provide a new avenue for the activation of apoptosis in diseased cells which refuse to die. The results from multiple rounds of selection are discussed.

BACKGROUND

Apoptosis, a form of programmed cell death, is essential to normal growth and development. It is implicated in a number of processes, including embryonic development and disease progression. It is in essence an organized method of cellular suicide. Due to the importance in timing, location, and progression of apoptosis, nature has developed a tight system of control over the various pathways that have been identified[1]. Abnormalities within this complex framework, e.g. inducing cell death when inhibition should occur or vice versa, can lead to various diseases and conditions. The objective is to research a new avenue for the activation of apoptosis in diseased cells which refuse to die.

At the cellular level, apoptosis commences when the cell receives extracellular signals on one or more of its death receptors[1]. Known pathways include Fas-mediated, TNF-related, and TRAIL-induced[1, 2]. The progression of apoptosis, which is far more complicated than simple ligand-binding, is an intricate system of checks and balances (Fig. 1) further complicated by numerous activations and inhibitions carried out by relevant macromolecules that often lead to conflicting signals. c-Myc is one such macromolecule[3]. It is capable of causing both cell cycle progression and apoptosis[3,4], and it is hindered in its apoptotic activity by Bcl-2 and c-FLIP[5,6]. While these proteins are certainly not the only macromolecules involved in the regulation of apoptosis, they are nonetheless significant and require further analysis.

c-Myc is a transcription factor encoded by the c-Myc gene, which is composed of three exons, the first of which is noncoding[3,7]. Two promoters, P1 and P2, effect 90-95% of all c-Myc transcription, and lead to the formation of 2.2 and 2.4 kb mRNAs and subsequently 64-67 kDa c-Myc proteins[3]. The protein is found in the nucleus and exhibits both nonspecific and site-

specific DNA binding at E-box domains (-CACGTG-)[8,9]. The RNA binding affinity of c-Myc protein is not well characterized, though it has been shown that RNA is not co-purified with c-Myc[10]. Nonetheless, it is likely that c-Myc can bind to RNA with the E-box complementary sequence[10].

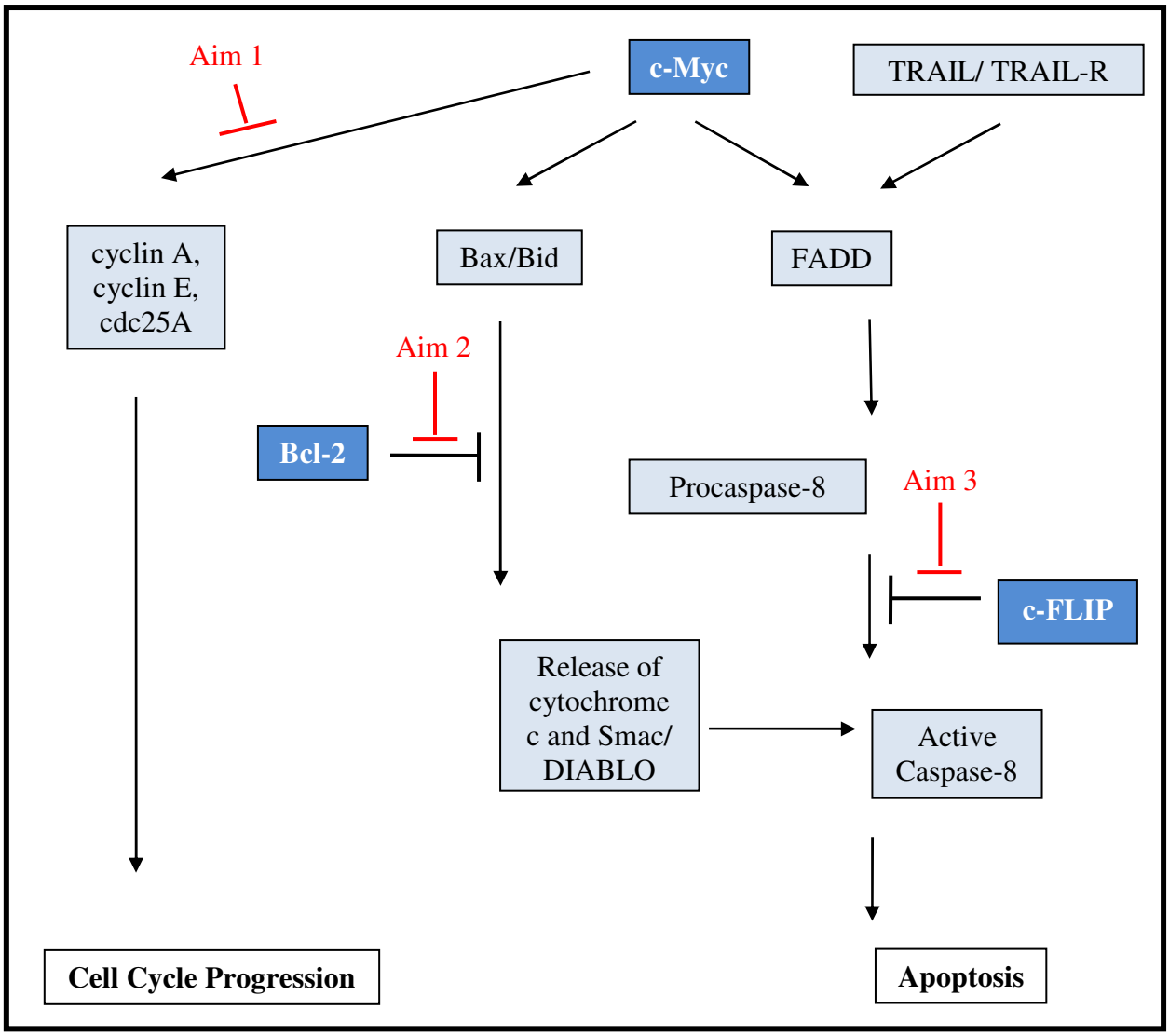


Figure 1: The role of c-Myc, Bcl-2, and c-FLIP in cell cycle progression and apoptosis, showing specific aims.

A surprising attribute of c-Myc is its instability—half-life of approximately thirty minutes[3]. So what accounts for their presence and activity in living systems? Stability is attained by dimerization with Max, a similar protein encoded by a different gene[10]. Myc-Max heterodimers confer tight binding to DNA, allowing for more adequate transcriptional regulation of genes involved in cell signaling[3,11,12].

The role of c-Myc in growth and development is not clear, and it has been shown that c-Myc can both induce and inhibit cell death (Fig. 2). Research in antisense oligonucleotide technology has revealed that a decrease in c-myc mRNA levels, and a subsequent decrease in c-Myc protein levels, results in growth inhibition and/or apoptosis[13,14]. The ambiguity in c-Myc function necessitates that any proposed method of inducing apoptosis in a Myc-related pathway, must take into consideration the ability of c-Myc to cause cell cycle progression. In addition, the proposed method should seek to understand the regulation of c-Myc-induced apoptosis. These considerations are the precise reason for the multifaceted approach sought here. Inhibition of one pathway is not enough to induce apoptosis—effective inducement can only be achieved by executing inhibition of all c-Myc-induced pathways.

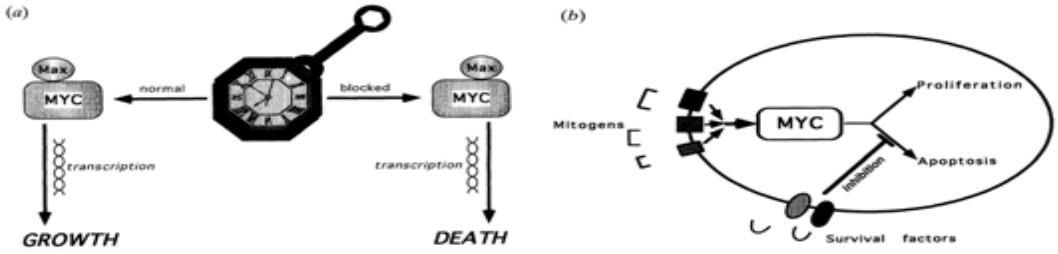


Figure 2: (a) Death arises because of conflict of signals. Under normal conditions, c-Myc promotes cell growth, but in the absence of serum, apoptosis results. (b) "Dual Signal" hypothesis. Under normal conditions, c-Myc induces both cell proliferation and apoptosis. Adapted from Evan *et al*.

The pathway in which c-Myc acts as an activator of apoptosis is the main thrust of this research. There is an important protein called Bcl-2 in this pathway, which inhibits c-Myc-activated Bax/Bid (Fig. 3), a heterodimer found in the mitochondrial outer membrane that increases permeability of the membrane to cytochrome c and Smac/DIABLO[15,16,17]. These serve as signals for the activation of caspase cascades, which unleash proteases, called caspases, resulting in the cleavage of proteins inside the cell, and ultimately cell death[15,16].

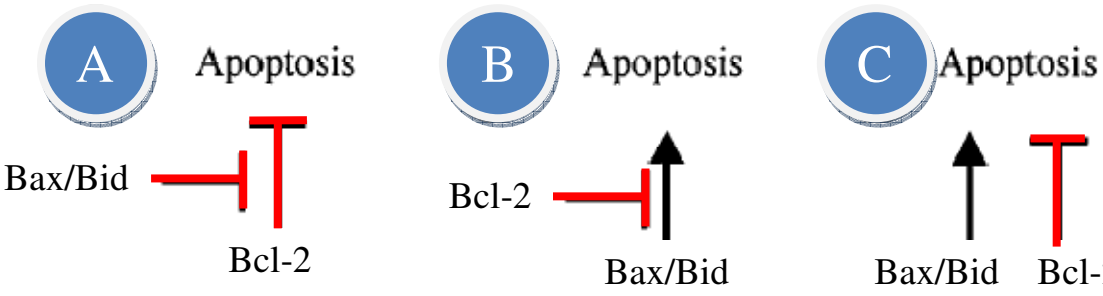


Figure 3: (A) Bax/Bid is upstream of Bcl-2. (B) Bax/Bid is downstream of Bcl-2. (C) Bax/Bid and Bcl-2 act independently to regulate apoptosis. Adapted from Chao and Korsmeyer.

Bcl-2 was the first of a family of pro-apoptotic and anti-apoptotic proteins to be characterized and is overexpressed in a number of cancers such as B cell lymphomas[16]. Studies on CED-9, a homologue of Bcl-2 found in *C. elegans*, demonstrate the upstream negative regulation characteristic of Bcl-2 function[18]. Inhibition of Bcl-2 is linked to tumor regression by apoptosis[19].

In a different, but related, pathway, tumor-necrosis-factor-related-apoptosis-inducing-ligand (TRAIL) causes cell death by binding to a death receptor, TRAIL-R, which recruits a molecule called Fas-associated via death domain (FADD) and causes activation of a caspase cascade[1,2,6]. TRAIL shows promise as a cancer therapeutic, but with one caveat[20]. The inhibitory protein of interest here, cellular FLICE-inhibitory protein (c-FLIP), antagonizes the transformation of procaspase-8 into its active form and, like Bcl-2, prevents cell death[20,21].

c-FLIP was first identified as a human cellular homolog of v-FLIP, a herpes virus FLIP capable of inhibiting death-receptor-mediated apoptosis[22]. Likewise, expression of c-FLIP is implicated in tumor progression[23]. The c-FLIP gene, located on chromosome 2, resembles both its homolog v-FLIP and procaspase 8, with death effector domains (DED) and caspase-like domains (Fig. 4). FLIPs inhibit apoptosis by competing for DED binding sites on FADD, thereby decreasing the likelihood of FADD-procaspase-8 binding.

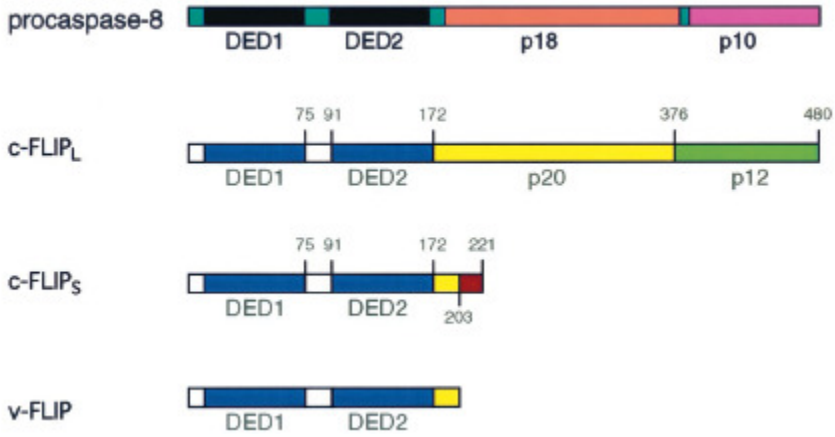


Figure 4: Structural similarities between caspase 8 and FLIP. Adapted from Krueger et al.

A number of studies has shown that expression of both Bcl-2 and c-FLIP result in the inhibition of Myc-mediated apoptosis[24,25,26,27,28,29]. Present research, especially cancer research, seeks to describe the imbalances in gene expression and/or protein formation that are characteristic of cells which refuse to die. Various technologies, such as siRNA, antisense oligonucleotides[30], antibodies[31], and aptamers, have been employed in the characterization of apoptosis pathways.

Characteristics that make aptamers powerful tools in diagnostics and therapeutics are their high specificity and high binding affinity[33,34]. Identification of nucleic acid aptamers against a number of pathogenic proteins has revealed a generalized inhibitory function. Aptamers against c-Myc, Bcl-2, and c-FLIP have not yet been identified. This research will employ the selection of RNA aptamers in understanding the roles of c-Myc, Bcl-2, and c-FLIP in cell death, as well as in the analyzing the interactions among these proteins. The ultimate goal is to discover a novel method for the inducement of cell death, with key applications for the treatment of diseases characterized by cell proliferation.

EXPERIMENTAL PROCEDURES

Pools—The selections were performed using the N70.01 and N35 random sequence pools, with 10^{13} complexity (provided by Bradley Hall and Shawn Piasecki, respectively). The synthesis of the pools is similar in technique to that described in Hesselberth *et al.* (2001).

Sequence—N70.01
5' – ATGCGTAGGCGTGTAACTTG - N70.01 - CACATTACAAGCTCGCCAGT - 3'
Forward primer—N70.01
5' – TTCTAATACGACTCACTATAGGATGCGTAGGCGTGAACTTG - 3'
Reverse primer—N70.01
3' - GTGTAATGTTCGAGCGGTCA - 5'.

Sequence—N35
5’ - CACAGCGGGACAGTTTAGC - N35 - GGTAGGTGGGTGCGTCTAAA - 3’
Forward primer—N35
5’ – GATAATACGACTCACTATAGGGCACAGCGGGACAGTTTAGC – 3’
Reverse primer—N35
3’- CCATCCACCCACGCAGATTT -5’

In Vitro Selection—Equimolar amounts (200 pmol) of RNA and protein were incubated at room temperature for 30 min following heat denaturation of the RNA (65 °C for 5 min, room temperature for 10 min). Binding and nonbinding species were separated by pushing the reaction through a nitrocellulose filter, with pores large enough to pass free nucleic acids but too small to pass those bound to protein. Binding species that were caught on the filter were thrice eluted with 50 mM EDTA, 5 M urea, and 0.3 M sodium acetate to give a final volume of 400 µL. Beginning with the third round, filter binders were excluded by passing the denatured RNA over a nitrocellulose filter prior to the addition of protein. Ethanol precipitation was performed to isolate the selected RNA, and reverse transcription was performed with SuperScript II reverse transcriptase (Life Technologies, Inc.) and the appropriate reverse primer. The cDNA products were amplified by polymerase chain reaction after the addition of the

forward primer and Taq DNA polymerase (Invitrogen). The dsDNA PCR amplified products were transcribed with the Ampliscribe T7 Transcription Kit (Epicentre). The reactions were incubated at 37°C for 6 hrs, denatured with 2X Denaturing dye, and run down a PAGE gel. The RNA was eluted a final time, and then quantified.

The dsDNA selection follows the same procedure, with the exception that reverse transcription and transcription were not performed.

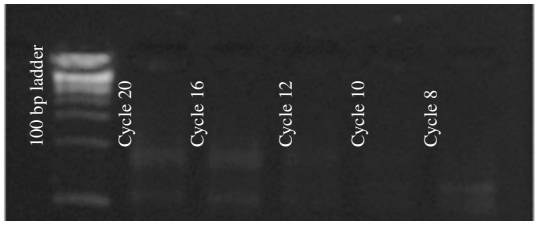
Additional Negative Selections—c-FLIP is commercially available as a GST-tagged protein. GST, or Glutathione S Transferase, is often used in the purification of proteins. Its presence will require the usage of negative selections to exclude RNA species that bind to GST and not to c-FLIP. This will be achieved by first incubating the RNA pool with GST, collecting the flow-through containing RNA species that do not bind to GST, and finally incubating with GST-c-FLIP. Negative selection against GST will begin with round three.

Binding Assays—Following six and ten rounds of selection, the percent binding of the RNA pool was ascertained. The dsDNA from rounds one, three, four, five, six, eight, and ten were transcribed using a radioactive nucleotide, [a-32P]ATP (3000 Ci/mmol, NEN Life Science Products). Elution and quantification proceeded as usual. The binding reactions were passed simultaneously over two filters, nitrocellulose and nylon. The percent binding is calculated by the relative amounts of radiation present on each filter. All binding is calculated above background binding, which is related to the amount of radiation present for the negative control containing nucleic acids and no protein.

RESULTS AND DISCUSSION

As previously described, there is some uncertainty in the RNA binding abilities of the transcription factor c-Myc. Two rounds of selection were performed using the N70.01 pool RNA, but a gel of the PCR products showed no definable bands, which indicated that N70.01 pool RNA did not bind to the protein. Though these results strongly suggest that c-Myc lacks RNA binding characteristics, it is entirely possible that binding does occur but is transient and weak.

For the first round of selection, 1.3 µg of N70.01 pool RNA (approximately 10^{13} molecules in 13.5 µL) was incubated with an equimolecular amount of protein (approximately 10^{12} molecules in 2 µL). The PCR cycle course gel (included below) showed overamplification at cycle 8; thus, large scale PCR was carried out to six cycles. The DNA was quantified (4793.1 ng/µL; 1:10 dilution gave 556.2 ng/µL), and a recalculation gave 1.3 µg in 2.34 µL to be used in the next round.

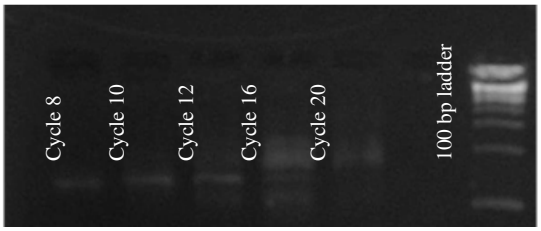


Round 1 c-Myc

The second round of selection was not as successful. Difficulties arose in reverse transcription of RNA. The resuspension of the precipitated cDNA prior to reverse transcription was successful, though the reverse transcription separated into two layers, the bottom milky white and the top clear, after a week at -20°C. Subsequent PCR showed no amplification. It is possible that the salts (sodium acetate) used in ethanol precipitation crashed out of solution, or

the sample became contaminated. Alternatively, it may be that the cDNA was not properly resuspended (e.g. overdried nucleic acids fail to dissolve completely).

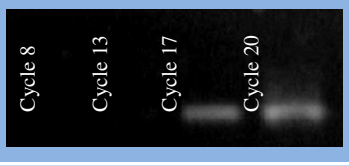
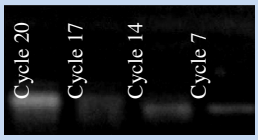
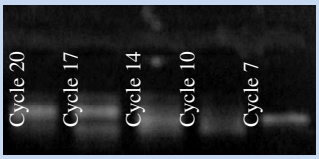
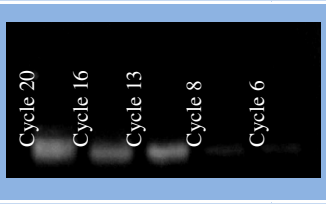
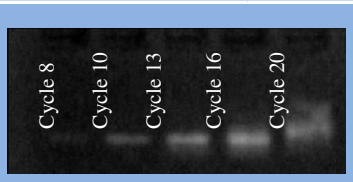
The cycle course from one round for Bcl-2 is shown below. The concentrations from RNA quantification following large scale amplification to cycle 7 and cycle 13, respectively for rounds 1 and 2, are found in Table 1.



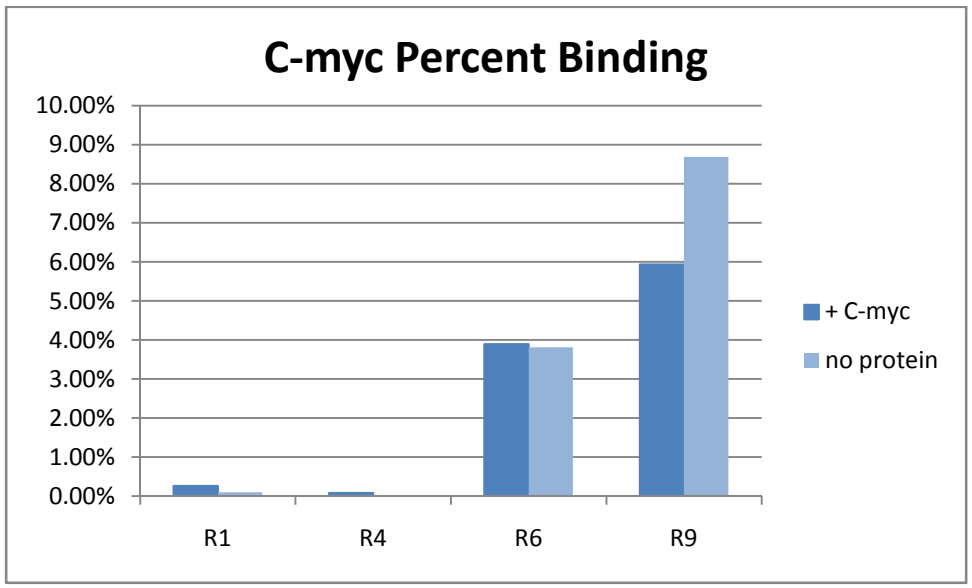
Round 1 Bcl-2

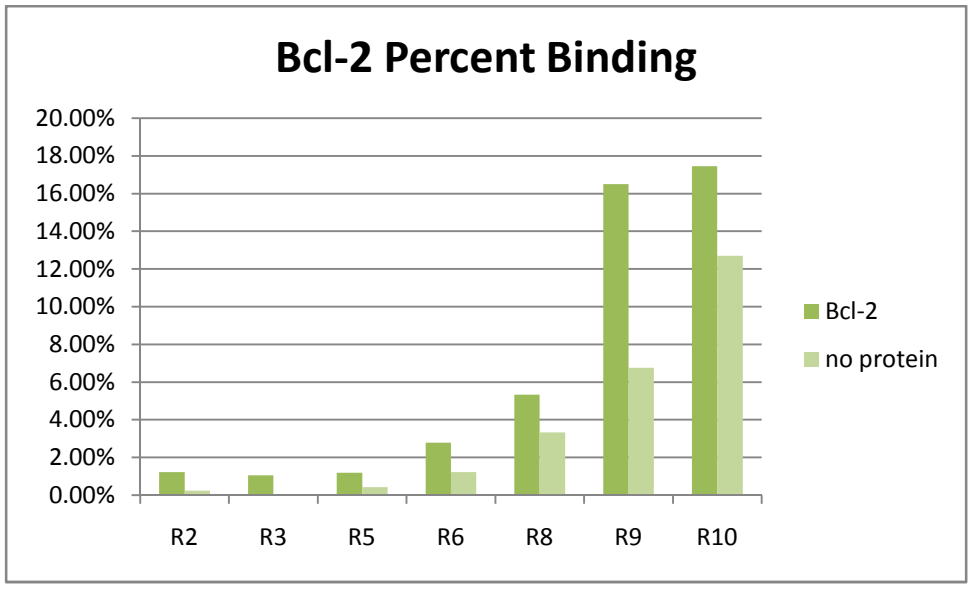
Table 1	Round 1 (μM)	Round 2 (μM)
Bcl-2	30.36	20.02

Selections against c-Myc and Bcl-2 were reevaluated following two rounds of selection with N70.01 pool RNA. A shorter random region was used instead, and ten rounds of selection were performed with N35 pool RNA. The results were promising, as expected. Table 2 shows PCR cycle courses and RNA quantifications from the first eight rounds.

Table 2	c-Myc		Bcl-2	
	PCR cycle course	RNA (uM)	PCR cycle course	RNA (uM)
Round 1		43.05		81.39
Round 2		44.67		40.32
Round 3		17.01		29.64
Round 4		45.68		78.13
Round 5		24.39		16.54
Round 6		28.2		22.2
Round 7		42.2		43.13
Round 8		24.47		28.12

The results from ten rounds of selection were assayed for percent binding of target protein to N35 pool. Significant results are demonstrated in the assay of round 9 binding for Bcl-2, which indicates good binding above background as well as good binding relative to an identical assay without protein. Cloning and sequencing of round 9 of selections against Bcl-2 may provide promising results for the identification of specific aptamers against Bcl-2; these steps are currently being done.





BUDGET

Protein	c-Myc (a.a. 1-262)	Bcl-2 minus C terminus	GST-c-FLIP (a.a. 1-481)
Supplier	Santa Cruz Biotechnology, Inc.	R&D Systems	Abnova Corporation
Catalog Number	sc-4084	827-BC-050	H00008837-P01
Molecular Weight	65 kDa	27 kDa	78.8 kDa
Weight	50 µg	50 µg	25 µg
Volume	1 mL	335.6 µL	N/A
Concentration	0.77 µM	5.52 µM	N/A
Buffer	Supplied as 50 µg purified protein in PBS containing 5 mM DTT and 50% glycerol.	Supplied frozen as a 0.2 µm filtered solution in 10 mM HEPES (pH 8.0) and 0.1 M KCl and 20% glycerol.	Supplied in 50 mM Tris-Hcl, 10 mM reduced Glutathione, pH=8.0 in the elution buffer.
Price	$150	$350	$390
Number of Rounds Possible	3.85	9.25	1.59
Price per Round	$39.00	$37.79	$245.86
Quantity	3	1	0
Total Price	$450	$350	$0

c-FLIP was not be purchased because of the high price per round (a typical round should cost approximately $50). Selections against this protein were not carried out because it could not be obtained from other research labs.

CONCLUSION

By virtue of pool nucleic acid folding complexity, it is expected that aptamers will be successfully selected against each protein target. However, the selection of aptamers that exhibit a significant percentage of binding to each protein target is dependent on the extent of each protein's ability to bind RNA. In the case of low binding affinity, more rounds of selection were carried out, and percentage binding was assessed again.

Once sufficient binding percentages were achieved, DNA from round 9 of selection against Bcl-2 was cloned—this process is ongoing. It is expected that potential aptamers will induce cell death, though their activities will have to be evaluated in a number of ways. For example, the aptamers can be placed in cancer cells to assess their effect on cell death. Both direct and indirect means of inhibition can be employed. The former method can be carried out by direct use of inhibitory aptamers and the latter by conjugation of toxins, proteases, and other apoptosis-inducing molecules to aptamers. Additionally, these aptamers may be used as biomarkers for various conditions.

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